REMARKS

Claims 1-21 are currently pending. These claims are cancelled in favor of new claims 22-58. Support for claim 22 which corresponds substantially to prior claim 1 can be found throughout the application as originally filed, *inter alia*, in claims 1, 9, and 10 as originally filed, in paragraph 14, line 3; in paragraph 15, line 7; in paragraph 45; in paragraph 65; and in paragraphs 68-70. New claims 23-58 find support throughout the originally filed application, *inter alia*, in original claims 1, 3, 4, 14, 15, 18, 19, and 21; and in paragraphs 11, 12, 64, and 66-70. Accordingly, Applicants submit that no new matter has been introduced into the application by way of the instant claim amendment and new claims.

Claims 1-21 are cancelled herein without prejudice or disclaimer as to the subject matter of the cancelled claims. Applicants respectfully reserve the right to pursue the subject matter of the cancelled claims in one or more continuation or divisional applications.

Rejections

35 U.S.C. § 102(b)

Claims 1, 4-9, and 11-13 were rejected under 35 U.S.C. § 102(b) as allegedly anticipated by the disclosure of Robinson *et al* (U.S. Patent No. 6,204,023)(hereinafter "Robinson").

Applicants respectfully disagree and traverse this rejection.

The Office Action states that Robinson discloses a method of producing a secreted heteromultimeric protein which is an antibody, in which a first cell which is haploid is transformed with a first plasmid encoding a light chain gene, and a second haploid cell is transformed with a second plasmid encoding a heavy chain gene, and the cells are mated to form a diploid cell, which secretes the heteromultimeric antibody protein (*citing* col. 15, line 38 - col. 16, line 55).

Under 35 U.S.C. § 102, the Patent Office bears the burden of presenting at least a prima facie case of anticipation. In re King, 801 F.2d 1324, 1326 (Fed. Cir. 1986). Anticipation requires that a prior art reference disclose, either expressly or under the principles of inherency, each and every element of the claimed invention. Celeritas Tech., Ltd., v. Rockwell Int'l Corp., 150 F.3d 1354, 1361 (Fed. Cir. 1998). The prior art reference must disclose all of the claim

elements arranged or combined in the same way as recited in the claim. Net MoneyIN, Inc. v. VeriSign, Inc., 545 F.3d 1359, 1369 (Fed. Cir. 2008). "In addition, the prior art reference must be enabling." Akzo N.V. v. U.S. International Trade Commission, 808 F.2d 1471, 1479 (Fed. Cir. 1986), cert. denied, 482 U.S. 909 (1987). That is, the prior art reference must sufficiently describe the claimed invention so as to have placed the public in possession of it. In re Donohue, 766 F.2d 531, 533 (Fed. Cir. 1985). Such possession is effected only if one of ordinary skill in the art could have combined the disclosure in the prior art reference with his/her own knowledge to make the claimed invention. Id.

Applicants note that the claims as amended herein require, in pertinent part, the production of stable diploid *Pichia* yeast cells capable of the assembly, expression and secretion of a heteromultimeric polypeptide into a culture medium. (As noted above, in order to expedite prosecution the pending claims are now all directed to methods of producing heteropolymeric polypeptides in *Pichia*). Applicants submit that Robinson does not contemplate the use of *Pichia* yeast cells as required by new independent claim 22. Furthermore, for reasons discussed in greater detail below with respect to the obviousness rejections, Robinson does not contemplate the production of stable diploid *Pichia* yeast cells, and certainly not those capable of the assembly, expression and secretion of a heteromultimeric polypeptide into a culture medium.

Because Robinson does not teach each and every element of the claimed invention, Applicants submit that Robinson does not anticipate the claimed invention. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection under 35 U.S.C. § 102(b) based on Robinson as this reference does not fairly teach or suggest the use of diploidal *Pichia* to express heteropolymeric polypeptides as set forth in claims 22-58.

35 U.S.C. § 103(a)

A. Claims 1-9, 11-13, and 15 were rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Robinson in view of International Publication No. WO 00/23579 to Choudary *et al* (hereinafter "Choudary").

Applicants respectfully disagree and traverse this rejection.

The Office Action cites to Robinson, noting that Robinson does not disclose "... a specific yeast strain, i.e., *Pichia* ... and inducible promoters ..." The Office Action cites to

Choudary for the expression of antibodies in *Pichia* strains. The Office Action also cites to Choudary for the use of inducible promoters. According to the Office Action, it would have been obvious to one of ordinary skill in the art to have used *Pichia* strains disclosed by Choudary in the method disclosed by Robinson, since according to the Office Action Robinson generally discloses that any yeast strain may be used, and it is purported that Choudary teaches advantages for using *Pichia* for the production of antibodies. *See* Office Action, pages 3-4.

Under 35 U.S.C. § 103, the Patent Office bears the burden of establishing a *prima facie* case of obviousness. In re Fine, 837 F.2d 1071, 1074 (Fed. Cir. 1988). There are four separate factual inquiries to consider in making an obviousness determination: (1) the scope and content of the prior art; (2) the level of ordinary skill in the field of the invention; (3) the differences between the claimed invention and the prior art; and (4) the existence of any objective evidence, or "secondary considerations," of non-obviousness. Graham v. John Deere Co., 383 U.S. 1, 17-18 (1966); see also KSR Int'l Co. v. Teleflex Inc., 127 S. Ct. 1727 (2007). An "expansive and flexible approach" should be applied when determining obviousness based on a combination of prior art references. KSR, 127 S. Ct. at 1739. However, a claimed invention combining multiple known elements is not rendered obvious simply because each element was known independently in the prior art. Id. at 1741. Rather, there must still be some "reason that would have prompted" a person of ordinary skill in the art to combine the elements in the specific way that he or she did. Id.; In re Icon Health & Fitness, Inc., 496 F.3d 1374, 1380 (Fed. Cir. 2007). Also, modification of a prior art reference may be obvious only if there exists a reason that would have prompted a person of ordinary skill to make the change. KSR, 127 S. Ct. at 1740-41.

As noted previously, Robinson does not contemplate the production of stable diploid *Pichia* yeast cells capable of the assembly, expression and secretion of a heteromultimeric polypeptide into a culture medium. Furthermore, Applicants submit that the combination of Robinson and Choudary does not render the claimed invention obvious.

The Office Action states that Robinson generally discloses that any yeast strain may be used. Applicants respectfully disagree, and submit that the prophetic teachings of Robinson (relating to mating of haploid yeast strains that respectively comprise heavy and light chain antibody sequences to produce diploidal yeast that allegedly will be capable of expressing and secreting authentic antibodies) should be limited to *Saccharomyces*, or at most yeast with mating and ploidy properties very similar thereto. Robinson only mentions *Saccharomyces*, and

contains no mention of *Pichia* or any other similar methylotrophic yeast. More specifically, Robinson only contains the following prophetic disclosure relating to the expression of antibodies in diploidal yeast:

> ... (3) The light and heavy chain genes are each attached to a yeast promoter and terminator sequence on separate plasmids each containing different selective markers as described in (2) above. A yeast mating type "a" strain defective in the selective markers found on the light and heavy chain expression plasmids (trp1 and ura3 in the above example) is transformed with the plasmid containing the light chain gene by selection for one of the two selective markers (trp1 in the above example). A yeast mating type "alpha" strain defective in the same selective markers as the "a" strain (i.e. tri1 and ura3 as examples) is transformed with a plasmid containing the heavy chain gene by selection for the alternate selective marker (i.e. ura3 in the above example). The "a" strain containing the light chain plasmid (phenotype: Trp^+ Ura^- in the above example) and the strain containing the heavy chain plasmid (phenotype: Trp^- Ura^+ in the above example) are mated and diploids are selected which are prototrophic for both of the above selective markers (Trp^+ Ura^+ in the above example)....

See Robinson, col. 16, lines 34-54.

Applicants submit that one of ordinary skill in the art would interpret the efficacy and efficiency of the proposed diploidization methods of Robinson to rely on the mating of two distinct mating strains, i.e., an "a" strain and an "alpha" strain, which respectively correspond to the male and female mating strains of *Saccharomyces*. However, unlike *Saccharomyces*, *Pichia* does not have readily or easily discernable male and female mating strains. Hence, Applicants submit that one of ordinary skill in the art reading the prophetic disclosure of Robinson would not reasonably intuit that that these teachings of Robinson are pertinent to *Pichia* or another yeast like *Pichia* which does not comprise readily discernable mating type strains.

Applicants further submit that the recitation of "stable" in the context of the present invention reflects the unexpected discovery that recombinant diploid *Pichia* yeast which have been produced by mating or spheroplast fusion and which have been genetically engineered to contain genetic constructs that provide for the expression of the respective subunits or chains that constitute a desired heterologous heteromultimeric polypeptide are stably maintained in said diploidal *Pichia* yeast after prolonged culture. Moreover, these stable diploid *Pichia* cells secrete the heteromultimeric polypeptide at unexpectedly high concentrations (i.e., 50 mg/L or more; *See also* Garcia-Martinez Declaration (attached herewith)) even after prolonged culturing.

Thus, the claimed invention also would not have been obvious to one of ordinary skill in the art because it could not have been expected that such diploids would stably retain the sequences required for antibody expression or that the desired heteromultimeric polypeptide would be efficiently secreted by the diploid *Pichia* cells. Applicants note that the mating cycle and mating forms of *Saccharomyces* and *Pichia* are quite dissimilar. Whereas wild-type homothallic haploid strains of *Saccharomyces* are unstable and rapidly mate to form diploids, this is not the case for *Pichia*. As mentioned previously, *Saccharomyces* has distinct haploid mating forms ("a" and "alpha"). By contrast, *Pichia* is an ascomycetous budding yeast that most normally exists in a vegetative haploid state. Unlike *Saccharomyces*, *Pichia* is most stable in its vegetative haploid state. In this regard, please refer to the following text, a copy of which is attached:

> *P. pastoris* diploid strains are unstable relative to haploid strains and will sporulate if subjected to the slightest stress (e.g., 1 or 2 wk on a YPD plate at room temperature). Thus, to maintain diploid strains, either transfer frequently to fresh plates or store frozen at -70°C. When working with these strains, check under the microscope frequently to be sure that strains are still diploid.

See Cregg *et al*, Methods in Molecular Biology, v. 103, *Pichia* Protocols: pp. 17-25, note 5 on page 25, and also p. 17, first paragraph extending to page 18 (1998).

Because *Pichia* has been reported to be most stable in its haploidal state (see above), Applicants submit that one of ordinary skill in the art would understand Cregg *et al* as teaching away from the use of diploidal *Pichia* for the expression and recovery of desired heteromultimeric polypeptides in a production method as claimed, since a reasonable expectation would have been that diploidal strains of *Pichia* would likely revert to their haploidal state upon prolonged culturing. Furthermore, Applicants submit that it could not have been reasonably anticipated that diploid *Pichia* would stably maintain expression constructs providing for the efficient expression of a desired heteromultimeric polypeptide into a culture medium.

The cited Choudary reference does not cure the deficiencies of Robinson. Applicants note that while Choudary suggest the use of *Pichia* and other yeast to express immunoglobulins and to recover the same, Choudary does not teach or suggest the use of diploid *Pichia* in their methods. Moreover, as Choudary does not even mention ploidy, Applicants submit that one of

ordinary skill in the art would have reasonably concluded that the *Pichia* strains used for antibody expression and recovery in Choudary are haploid.

As stated previously, the inventors unexpectedly discovered that recombinant diploid *Pichia* yeast can be used for the expression and secretion of heteromultimeric polypeptides such as antibodies at unexpectedly high expression levels, and that these levels are stably maintained even after significant culturing (i.e., more that 20 doublings). Moreover, it has been found that these stable diploid *Pichia* cells secrete the heteromultimeric polypeptide at unexpectedly high concentrations (i.e., 50 mg/L or more; *See also* Garcia-Martinez Declaration (attached herewith)) even after prolonged culturing.

In contrast, Choudary states the following with respect to observed levels of antibody expression using their haploid *Pichia* expression system:

> The kinetics of antibody production/secretion were followed by withdrawing portions of the culture at various intervals of methanol-induction of the clones and determining the antibody levels by slot-immunoblot analysis. As seen in Figure 6, anti-dioxin antibody was detectable in culture medium between 12 hours and 120 hours of induction, with highest levels of about 10 to 36 mg l^{-1} detected between 72 and 108 hours.

See Choudary, page 25, lines 1-8.

By contrast, it has been found that the stable diploid *Pichia* cells of the invention secrete the heteromultimeric polypeptide at unexpectedly high concentrations (i.e., 50 mg/L or more; *See also* Garcia-Martinez Declaration (attached herewith)) even after prolonged culturing, and even up to around 1000 mg/L.

It was non-obvious and unexpected at the time of filing that diploidal *Pichia* yeast would stably (over prolonged culture times) secrete large quantities of a mammalian multichain polypeptide into the culture medium. As discussed in the instant application, *Pichia* diploids remain stable for prolonged periods of time, and maintain their ability to secrete very large quantities of the multichain polypeptide into the culture medium.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) over Robinson in view of Choudary.

B. Claims 1-9, 11-14, and 16-20 were rejected under 35 U.S.C. § 103(a) as allegedly unpatentable over Robinson as applied to claims 1, 4-9, and 11-13, and further in view of the disclosure of Zamost (U.S. Patent No. 6,258,559)(hereinafter "Zamost").

The Office Action cites to Robinson for reasons previously noted. The Office Action cites to Zamost for the use of the GAP promoter and the use of minimal media for culturing and production of recombinant proteins. According to the Office Action, it would have been obvious to one of ordinary skill in the art to have modified the method of Robinson to utilize a known constitutive promoter such as GAP, since both references concern the production of recombinant proteins in yeast, and since the use of any particular promoter for the properties known to be associated with that promoter were well known in the art. The Office Action also states that it would have been obvious to culture *Pichia* using minimal media as disclosed by Zamost, in order to obtain benefits disclosed by Zamost which include better growth and yield. *See* Office Action, page 4.

Applicants respectfully disagree and traverse this rejection.

Applicants submit that Robinson does not teach or suggest all of the elements of the claims for the reasons set forth above regarding the obviousness rejection combining the Robinson and Choudary references. Applicants further submit that the Zamost reference does not cure the deficiencies of Robinson.

Applicants note that Zamost does not even mention ploidy. For the reasons set forth above with respect to Robinson and Choudary, Applicants submit that one of ordinary skill in the art would have reasonably concluded that the *Pichia* strains used in Zamost are haploid.

As stated previously, the inventors unexpectedly discovered that recombinant diploid *Pichia* yeast can be used for the expression and secretion of heteromultimeric polypeptides such as antibodies at unexpectedly high expression levels, and that these levels are stably maintained even after significant culturing (i.e., more that 20 doublings). Moreover, it has been found that these stable diploid *Pichia* cells secrete the heteromultimeric polypeptide at unexpectedly high concentrations (i.e., 50 mg/L or more; *See also* Garcia-Martinez Declaration (attached herewith)) even after prolonged culturing.

Applicants reiterate that it was non-obvious and unexpected at the time of filing that diploidal *Pichia* yeast would stably (over prolonged culture times) secrete large quantities of a mammalian multichain polypeptide into the culture medium. As discussed in the instant

application, *Pichia* diploids remain stable for prolonged periods of time, and maintain their ability to secrete very large quantities of the multichain polypeptide into the culture medium.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) as allegedly unpatentable over Robinson in view of Zamost.

35 U.S.C. § 112, 1st Paragraph

Claims 21 was rejected under 35 U.S.C. § 112, 1st paragraph, as allegedly lacking enablement. More specifically, it is asserted in the Office Action that in light of the state of the prior art, it is not routine to produce 100 mg/l of complete antibodies in recombinant yeast.

Applicants respectfully disagree and traverse this rejection.

As an initial matter with respect to this rejection, Applicants note that claim 21 has been cancelled herein. Nevertheless, new claims 42-44 recite that the level of the biologically active heteromultimeric polypeptide in the culture medium is at least 100 mg/l (500 mg/l and 1000 mg/l in claims 43 and 44, respectively).

Applicants provide herewith the Declaration of Dr. Leon Garcia-Martinez under 37 C.F.R. § 1.132. Dr. Garcia-Martinez is employed by Alder Biopharmaceuticals, Inc., the owner of all right, title and interest in the instant application. In his Declaration, Dr. Garcia-Martinez provides experimental results regarding the expression of several antibodies in diploid *Pichia pastoris* cells. As can be seen from the results, and in the opinion of Dr. Garcia-Martinez, one of ordinary skill in the art following the teachings of the instant application as of October 22, 2003, and using the well known techniques for delivery of feed to diploid *Pichia pastoris* yeast cells (transformed to express and secrete into the culture medium a complete full length monoclonal antibody) would have been able, absent undue experimentation, to reproducibly achieve expression levels of at least 100 mg/l or higher in the fermentation supernatant following a reasonable fermentation time. *See, for example*, Garcia-Martinez Declaration, paragraph 13. It is submitted that this Declaration demonstrates that the claimed methods are adequately described and enabled by the teachings in the as-filed application and demonstrates that one skilled in the art practicing the disclosed methods and using diploidal *Pichia* produced according to the invention along with known fermentation methods and media containing constituents well known in the relevant art would be able to reproducibly obtain the results which are recited in the

claims absent undue experimentation. Moreover, while the Declarant notes that some amount of optimization may result in enhanced expression levels, he further opines that in his opinion and based on his familiarity with such fermentation processes that such optimization would have been within the abilities of one of ordinary skill in the art as of October 22, 2003, and would not rise to the level of "undue experimentation." *See, for example*, Garcia-Martinez Declaration, paragraph 12.

Therefore, Applicants submit that the statement in the Office Action that it is not routine to produce 100 mg/l of complete antibodies in recombinant yeast further supports Applicants' non-obviousness argument set forth previously and does not contradict Applicant's traversal of the enablement rejection. Rather, while such results are truly unexpected at the date of the invention they are obtainable absent undue experimentation using the novel methods and materials (stable transformed diploidal strains) disclosed herein. This is evidenced by the concentrations of antibody production demonstrated in the Garcia-Martinez Declaration which are reproducibly obtained using different fermentation methods (feeding methods) then known in the art. Based thereon, Applicants respectfully request reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, 1st paragraph as allegedly lacking enablement.

35 U.S.C. § 112, 2nd Paragraph

Claims 10, 16, 17, and 20 were variously rejected under 35 U.S.C. § 112, 2nd paragraph, as allegedly indefinite in the recitation of certain terms and for antecedent basis. Applicants submit that the rejections under 35 U.S.C. § 112, 2nd paragraph are rendered moot by way of claim cancellation. Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, 2nd paragraph.

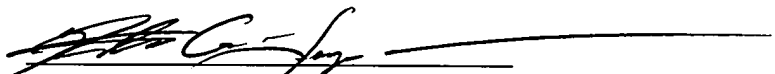
Conclusion

An indication of allowance of all claims is earnestly solicited. Early notification of a favorable consideration is respectfully requested.

Respectfully submitted,

HUNTON & WILLIAMS LLP

Dated: September 18, 2009

By: 

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David R. Higgins and James M. Cregg, Methods in Molecular Biology,
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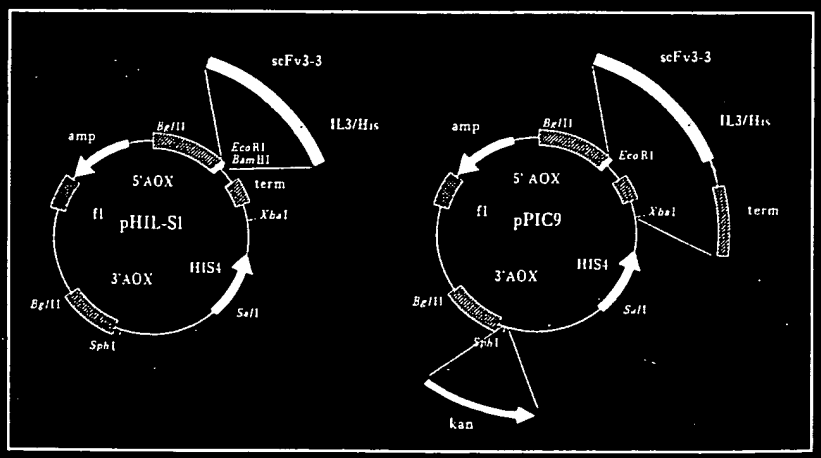

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2

Classical Genetic Manipulation

James M. Cregg, Shigang Shen, Monique Johnson, and Hans R. Waterham

1. Introduction

A significant advantage of *Pichia pastoris* as an experimental system is the ability to bring to bear readily both classical and molecular genetic approaches to a research problem. Although the recent advent of yeast molecular genetics has introduced new and exciting capabilities, classical genetics remains the approach of choice in many instances. These include: the generation of mutations in previously unidentified genes (mutagenesis); the removal of unwanted secondary mutations (backcrossing); the assignment of mutations to specific genes (complementation analysis); and the construction of strains with new combinations of mutant alleles. In this chapter, these and other methods for genetic manipulation of *P. pastoris* are described.

To comprehend the genetic strategies employed with *P. pastoris*, it is first necessary to understand basic features of the life cycle of this yeast *(1,2)*. *P. pastoris* is an ascomycetous budding yeast that most commonly exists in a vegetative haploid state (**Fig. 1**). On nitrogen limitation, mating occurs and diploid cells are formed. Since cells of the same strain can readily mate with each other, *P. pastoris* is by definition homothallic. (However, it is probable that *P. pastoris* has more than one mating type that switches at high frequency and that mating occurs only between haploid cells of the opposite mating type. In the related yeast *Pichia methanolica* [a.k.a. *Pichia pinus*], the existence of two mating types has been demonstrated by the isolation of mating type interconversion mutants, which are heterothallic *[3,4]*.) After mating, the resulting diploid products can be maintained in that state by shifting them to a standard vegetative growth medium. Alternatively, they can be made to proceed through meiosis and to the production of asci containing four haploid spores.

From: *Methods in Molecular Biology, Vol. 103:* Pichia *Protocols*
Edited by: D. R. Higgins and J. M. Cregg

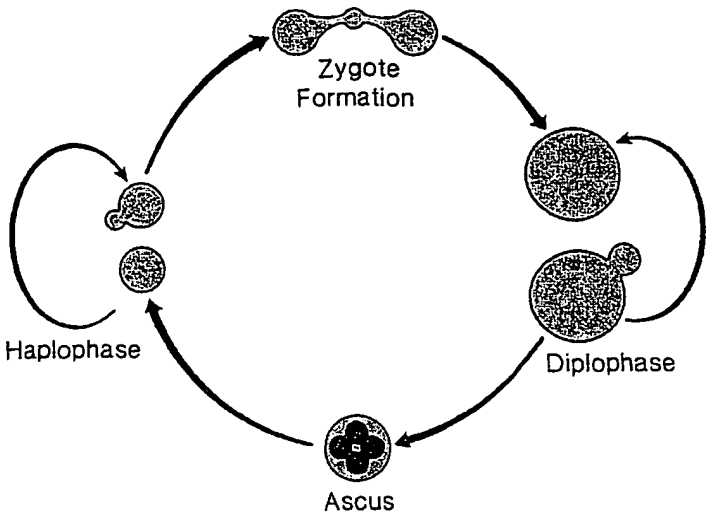

Fig. 1. Diagram of the *P. pastoris* life cycle.

The key feature of the *P. pastoris* life cycle that permits genetic manipulation is its physiological regulation of mating. *P. pastoris* is most stable in its vegetative haploid state, a great advantage in the isolation and phenotypic characterization of mutants. (In wild-type homothallic strains of *Saccharomyces cerevisiae*, the reverse is true: haploid cells are unstable and rapidly mate to form diploids *[5]*.) To cross *P. pastoris*, selected pairs of complementarily marked parental strains are mixed and subjected to nitrogen limitation for a time period sufficient to initiate mating. The strains are then shifted to a nonlimiting medium supplemented with a combination of nutrients that select for growth of hybrid diploid strains, and against the growth of the haploid parental strains and self-mated diploid strains. To initiate meiosis and sporulation, diploid strains are simply returned to a nitrogen-limited medium.

2. Materials

2.1. Strains and Media

All *P. pastoris* strains are derivatives of the wild-type strain NRRL-11430 (Northern Regional Research Laboratories, Peoria, IL). Auxotrophically marked strains are convenient for selection of diploid strains, and a collection of such strains is listed in **Table 1**. The identity of the biosynthetic genes affected in these strains is known for only three of the mutant groups: *his4*, histidinol dehydrogenase; *arg4*, argininosuccinate lyase; and *ura3*, orotidine-5'-phosphate decarboxylase. The *ura5* group strains are resistant to 5-fluoroorotic acid and

Table 1
Auxotrophic Mutants of *P. pastoris*

Representative strain	Geneotype	Representative strain	Geneotype
JC233	*his1*	JC239	*met1*
JC234	*his2*	JC240	*met2*[a]
GS115	*his4*	JC241	*met3*[a]
		JC242	*met4*[a]
JC247	*arg1*		
JC248	*arg2*	JC220	*ade1*
GS190	*arg4*	JC221	*ade2*
		JC222	*ade3*
JC235	*lys1 his4*	JC223	*ade4*
JC236	*lys2 his4*	JC224	*ade5*
JC237	*lys3*	JC225	*ade6*
		JC226	*ade7 his4*
JC251	*pro1*		
JC252	*pro2*	JC254	*ura3*
		JC255	*ura5*

[a]Mutants in these groups will grow when supplemented with either methionine or cysteine.

therefore, are thought to be defective in the homolog of the *S. cerevisiae* orotidine-5'-phosphate pyrophosphorylase gene (*URA5*), mutants of which are also resistant to the drug *(6)*. *P. pastoris ade1* strains are pink and, therefore, may be defective in the homolog of the *S. cerevisiae ADE1* (PR-aminoimidazolesuccinocarboxamide synthase) or *ADE2* (PR-aminoimidazole carboxylase) genes *(7)*.

P. pastoris strains are grown at 30°C in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or YNB medium (0.67% yeast nitrogen base without amino acids) supplemented with either 0.4% glucose or 0.5% methanol. Amino acids and nucleotides are added to 50 µg/mL as required. Mating (sporulation) medium contains 0.5% sodium acetate, 1% potassium chloride, and 1% glucose. Uracil-requiring mutants are selected on 5-FOA medium, which is composed of YNB glucose medium supplemented with 50 µg/mL uracil and 750 µg/mL 5-fluoroorotic acid (PCR, Inc., Gainesville, FL). For solid media, agar is added to 2%.

2.2. Reagents for Mutagenesis

1. 1 mL of a 10 mg/mL solution of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Sigma Chemical, St. Louis, MO) in acetone, stored frozen at -20°C (*see* **Note 1**).
2. 1 L mutagenesis buffer: 50 m*M* potassium phosphate buffer, pH 7.0.
3. 2 L 10% Na thiosulfate.

3. Methods

3.1. Long-Term Strain Storage

1. Viable *P. pastoris* strains are readily stored frozen for long periods (>10 yr). For each strain to be stored, pick a single fresh colony from a plate containing a selective medium, and inoculate the colony into a sterile tube containing 2 mL of YPD liquid medium.
2. After overnight incubation with shaking, transfer 1.2 mL of the culture to a sterile 2.0-mL cryovial containing 0.6 mL of glycerol. Mix the culture and glycerol thoroughly, and freeze at –70°C.
3. To resurrect a stored strain, remove the cryovial from the freezer, immediatley plunge a hot sterile inoculation loop into the frozen culture, transfer a few microliters of the culture from the loop to an agar plate containing an appropriate medium, and immediately return the culture to the freezer (*see* **Note 2**).

3.2. NTG Mutagenesis

1. Inoculate the strain to be mutagenized into a 10-mL preculture of YPD, and incubate with shaking overnight (*see* **Note 3**).
2. On the next morning, dilute the preculture with fresh medium, and maintain it in logarithmic growth phase (OD_{600} < 1.0) throughout the day.
3. In late afternoon, use a portion of the preculture to inoculate a 500-mL culture of YPD medium in a baffled Fernbach culture flask (or alternatively two 250-mL cultures in 1-L baffled culture flasks) to an OD_{600} of approx 0.005 and incubate with shaking overnight (*see* **Note 4**).
4. On the following morning, harvest the culture at an OD_{600} of approx 1.0 by centrifugation at 5000*g* and 4°C for 5 min, and suspend the culture in 100 mL of cold sterile mutagenesis buffer.
5. Determine the density of the culture and transfer 100 OD_{600} U to each of four 250-mL sterile plastic centrifuge bottles.
6. Adjust the volume in each bottle to 100 mL with the same buffer, and wash the cells once more by centrifugation and resuspension in 50 mL of the buffer.
7. Add aliquots of 100, 200, and 400 µL of the NTG solution to each of three cultures, and hold the fourth as an untreated control. Incubate the cultures for 1 h at 30°C, and stop the mutagenesis by adding 50 mL of a 10% Na thiosulfate solution to each culture.
8. Wash each mutagenized culture by centrifugation once with 100 mL of mutagenesis buffer and once with 100 mL of YPD medium, and resuspend each in 150 mL of YPD medium.
9. Remove 100-µL samples of each culture, prepare 100- and 10,000-fold serial dilutions of each, and spread 100-µL aliquots of the dilutions on YPD plates to determine the percentage of cells that have survived mutagenesis in each culture. Optimal survival rates are between 2 and 20% of the untreated control culture.
10. Transfer each mutagenized culture to a 500-mL shake flask, and allow cells to recover for 4 h at 30°C with shaking.

11. Concentrate the final cultures by centrifugation, and resuspended in 15 mL of YPD medium in 30% glycerol.
12. Place aliquots of 0.5 mL of NTG-treated samples into sterile microcentrifuge tubes or cryovials, and store frozen at –70°C for future use.
13. In preparation for screening mutagenized cultures, thaw a tube of each, serially dilute in sterile water, spread on a nonselective medium, such as YPD, YNB glucose, or other suitable medium, and determine the concentration of culture required to produce 500–1000 colonies/plate.
14. Screen for mutants by replica plating onto sets of plates containing appropriate diagnostic media.

3.3. Selection for Uracil Auxotrophs Using 5-Fluoroorotic Acid

5-Fluoroorotic acid (5-FOA), a uracil biosynthetic pathway analog, is metabolized to yield a toxic compound by certain enzymes in the pathway *(8)*. As a result, organisms that are prototrophic for uracil synthesis (Ura^+) are sensitive to 5-FOA, whereas certain Ura^- auxotrophs cannot metabolize the drug and, thus, are resistant to it. Selection for 5-FOA-resistant strains of *P. pastoris* is a highly effective means of isolating Ura^- mutants affected in either of two Ura pathway genes. One of these genes, *URA3*, encodes orotidine-5'-phosphate decarboxylase. The other is likely to be the homolog of the *S. cerevisiae* orotidine-5'-phosphate pyrophosphorylase gene (*URA5*), since *ura5* mutants represent the other complementation group selected by 5-FOA in this yeast.

1. To select for Ura^- strains of *P. pastoris*, spread approx 2 OD_{600} units (~5×10^7 cells) on a 5-FOA plate. Resistant colonies will appear after approx 1 wk at 30°C.
2. Test the 5-FOA-resistant colonies for Ura phenotype by streaking them onto each of two YNB glucose plates, one with and one without uracil. The highest frequency of Ura^- mutants is found in mutagenized cultures like those described above. However, Ura^- stains often exist at a low, but significant frequency within unmutagenized cell populations as well, and can be readily selected by simply suspending cells from a YPD plate in sterile water and spreading them onto a 5-FOA plate.
3. If it is necessary to determine which *URA* gene is defective in new Ura^- strains, the strains can either be subjected to complementation testing against the known *ura* mutants (**Table 1**) or transformed with a vector containing the *P. pastoris URA3* gene (*see* Chapters 3 and 7).

3.4. Mating and Selection of Diploids

The mating and selection of diploid strains constitute the core of complementation analysis, and are the first step in strain construction and backcrossing. Because *P. pastoris* is functionally homothallic, the mating type of strains is not a consideration in planning a genetic cross. However, since cells of the same strain will also mate, it is essential that strains to be crossed contain complementary markers that allow for the selective growth of crossed diploids,

and against the growth of self-mated diploids or parental strains. Auxotrophic markers are generally most convenient for this purpose, but mutations in any gene that affect the growth phenotype of *P. pastoris*, such as genes required for utilization of a specific carbon source (e.g., methanol or ethanol) or nitrogen source (e.g., methylamine), can be used as well.

1. To begin a mating experiment, select a fresh colony (no more than 1 wk old) of each strain to be mated from a YPD plate using an inoculation loop, and streak across the length of each of two YPD plates (**Fig. 2A**).
2. After overnight incubation, transfer the cell streaks from both plates onto a sterile replica plate velvet such that the streaks from one plate are perpendicular to those on the other.
3. Transfer the cross-streaks from the velvet to a mating medium plate, and incubate overnight to initiate mating.
4. On the next day, replica plate to an appropriate agar medium for the selection of complementing diploid cells. Diploid colonies will arise at the junctions of the streaks after approx 3 d of incubation (**Fig. 2B**). Diploid cells of *P. pastoris* are approximately twice as large as haploid cells and are easily distinguished by examination under a light microscope.
5. Colony-purify diploid strains by streaking at least once for single colonies on diploid selection medium (*see* **Note 5**).

3.5. Sporulation and Spore Analysis

Diploid *P. pastoris* strains efficiently undergo meiosis and sporulation in response to nitrogen limitation.

1. To initiate this phase of the life cycle, transfer freshly grown diploid colonies from a YPD plus glucose plate to a mating (sporulation) plate either by replica plating or with an inoculation loop, and incubate the plate for 3–4 d. After incubation, sporulated samples will have a distinctive tan color relative to the normal white color of vegetative *P. pastoris* colonies. In addition, spores and spore-containing asci are readily visible by phase-contrast light microscopy.
2. To analyze spore products by the random spore method, transfer an inoculation loop full of sporulated material to a 1.5-mL microcentrifuge tube containing 0.7 mL of sterile water, and vortex the mixture.
3. In a fume hood, add 0.7 mL of diethyl ether to the spore preparation, mix thoroughly, and leave standing in the hood for approx 30 min at room temperature. The ether treatment selectively kills vegetative cells remaining in spore preparations.
4. Serially dilute samples of the spore preparation (the bottom aqueous phase) to approx 10^{-4}, and spread 100-µL samples of each dilution onto a nonselective medium (e.g., YPD).
5. After incubation, replica plate colonies from plates that contain in the range of 100–1000 colonies onto a series of plates containing suitable diagnostic media. For example, to analyze the spore products resulting from a cross of GS115 (*his4*)

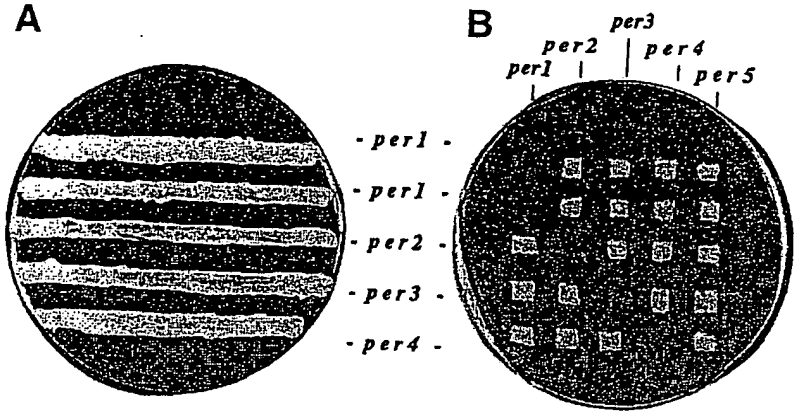

Fig. 2. Complementation analysis plates. (A) A YPD medium plate in which five methanol-utilization-defective strains have been streaked in preparation for complementation testing. (B) A YNB methanol medium plate on which complementing diploid strains have selectively grown.

and GS190 (*arg4*), appropriate diagnostic media would be YNB plus glucose supplemented with:

a. No amino acids;
b. Arginine;
c. Histidine; and
d. Arginine and histidine.

6. Compare or score the phenotype of individual colonies on each of the diagnostic plates, and identify ones with the desired phenotype(s).
7. For backcrossing or strain construction, select several colonies that appear to have the appropriate phenotype, streak for single colonies onto a nonselective medium plate, and retest a single colony from each streak on the same set of diagnostic medium-containing plates. This step is important since *P. pastoris* spores adhere tightly to one another, and colonies resulting from spore germination frequently contain cells derived from more than one spore. Another consequence of spore clumping is that markers appear not to segregate 2:2, but to be biased toward the dominant or wild-type phenotype. For example, in the GS115 (*his4*) × GS190 (*arg4*) cross described above, more His^+Arg^+ spore products will be apparent than the 25% expected in the population, and His^-Arg^- spore products will appear to be underrepresented.

3.6. Regeneration of Selectable Markers by Ectopic Gene Conversion

In *P. pastoris*, the number of genetic manipulations (e.g., gene replacements or gene knockouts) that can be performed on a single strain is constrained by

the limited number of selectable marker genes that are available. Since each new marker requires considerable effort to develop, a convenient means of regenerating previously used markers is sometimes useful. One general method takes advantage of the high frequency of homologous recombination events in diploid strains of *P. pastoris* undergoing meiosis *(9)*. In addition to expected recombination events between genes and their homologs at the normal loci on the homologous chromosome, recombination events also occur between genes and homologous copies located at other (ectopic) sites in the genome. Thus, a wild-type *P. pastoris* marker gene inserted into the *P. pastoris* genome at an ectopic location as part of a gene knockout construction can be meiotically stimulated to recombine with its mutant allele located at the normal locus. A frequent result of such events is an ectopic gene conversion in which the wild-type allele at the knockout site is converted to its mutant allele. Spore products that harbor a mutant allele-containing knockout construction are once again auxotrophic for the selectable marker gene, and can be identified by a combination of random spore and Southern blot analyses.

As an example of this strategy, we constructed a *P. pastoris* strain in which the alcohol oxidase genes *AOX1* and *AOX2* had been disrupted with DNA fragments containing the *S. cerevisiae ARG4* (*SARG4*) and *P. pastoris HIS4* (*PHIS4*) genes, respectively (**Fig. 3**, lane 3) *(9)*. This strain, KM7121 (*arg4 his4 aox1* :: *SARG4 aox2* :: *PHIS4*) cannot grow on methanol, but is prototrophic and therefore not easily transformed. Ectopic gene conversion events between the wild-type *HIS4* allele at *AOX2* and mutant *his4* alleles at their normal genomic loci were induced by crossing KM7121 with PPF1 (*arg4 his4 AOX1 AOX2*) (**Fig. 3**, lane 1), selecting for diploid strains on YNB methanol medium (**Fig. 3**, lanes 4–7) and sporulating the diploids. Approximately 5% of the resulting spores were, like the parent strain KM7121, unable to grow on methanol, but unlike KM7121, were auxotrophic for histidine. A Southern blot of the *AOX2* locus in these strains confirmed that in each the locus was still disrupted, but with a mutant *Phis4* allele (**Fig. 3**, lanes 13 and 14).

4. Notes

1. NTG is a powerful mutagen and a potent carcinogen. Therefore, great care should be exercised in handling this hazardous compound. Gloves, eye protection, and lab coat should be worn while working with NTG. The compound is most dangerous as a dry powder, and therefore, a particle filter mask should be worn when weighing out the powder. All materials that come in contact with NTG should be soaked overnight in a 10% solution of Na thiosulfate prior to disposal or washing.
2. Freezing kills approx 90% of the cells. However, once frozen, the remaining cells maintain their viability. Thus, it is critical not to allow the frozen culture to thaw, since approx 90% of the remaining viable cells will be killed with each round of freezing.

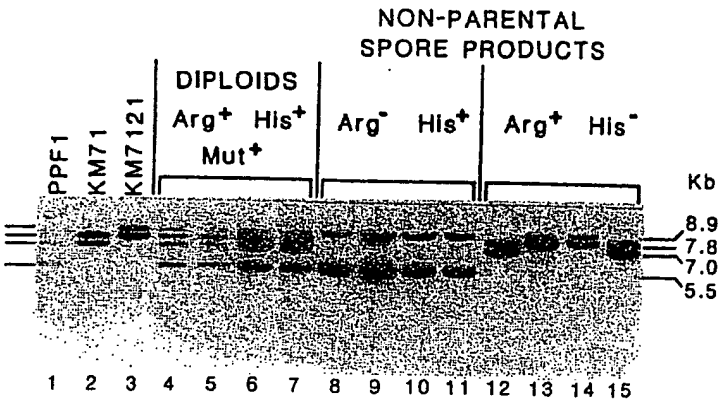

Fig. 3. Southern blot of selected strains resulting from the cross of KM7121 and PPF1. *Eco*RI-digested genomic DNA samples from the following strains are shown: lane 1, PPF1 (*arg4 his4 AOX1 AOX2*); land 2, KM71 (*arg4 his4 aox1* :: *SARG4 AOX2*); lane 3, KM7121 (*arg4 his4 aox1* :: *SARG4 aox2* :: *PHIS4*); lanes 4–7, diploid stains; lanes 8–11, Arg$^-$ His$^+$ Mut$^+$ nonparental spore products (*arg4 his4 AOX1 aox2* :: *PHIS4*); lanes 12 and 15, Arg$^+$ His$^-$ Mut^s nonparental spore products (*arg4 his4 aox1* :: *SARG4 AOX2*); lanes 13 and 14, Arg$^+$ His$^-$ Mut$^-$ ectopic gene conversion spore products (*arg4 his4 aox1* :: *SARG4 aox2* :: *Phis4*).

3. This procedure is a modified version of that described by Gleeson and Sudbury *(10)* and Liu et al. *(2)*. Alternative mutagenesis methods, such as with ethylmethane sulfonate or UV light, may also be effective with *P. pastoris* and are described in Rose et al. *(11)*, Spencer and Spencer *(12)*, and Sherman *(13)*.
4. The starting density of this culture can be adjusted to compensate for changes in the length of the incubation period. Adjust the density assuming that *P. pastoris* has a generation time of between 90 and 120 min at 30°C in YPD medium.
5. *P. pastoris* diploid strains are unstable relative to haploid strains and will sporulate if subjected to the slightest stress (e.g., 1 or 2 wk on a YPD plate at room temperature). Thus, to maintain diploid strains, either transfer frequently to fresh plates or store frozen at –70°C. When working with these strains, check under the microscope frequently to be sure that strains are still diploid.

References

1. Cregg, J. M. (1987) *Genetics* of methylotrophic yeasts, in *Proceedings of the Fifth International Symposium on Microbial Growth on C1 Compounds* (Duine, J. A. and Verseveld, H. W., eds.), Martinus Nijhoff, Dordrecht, The Netherlands, pp. 158–167.